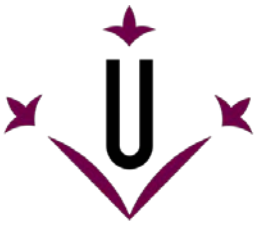

Heading date QTL in a spring x winter barley cross evaluated in Mediterranean environments

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Abstract

Heading date is a key trait for the adaptation of barley to Mediterranean environments. We studied the genetic control of flowering time under Northern Spanish (Mediterranean) conditions using a new population derived from the spring/winter cross Beka/Mogador. A set of 120 doubled haploid lines was evaluated in the field, and under controlled temperature and photoperiod conditions. Genotyping was carried out with 215 markers (RFLP, STS, RAPD, AFLP, SSR), including markers for vernalization candidate genes, HvBM5 (*Vrn-H1*), HvZCCT (*Vrn-H2*), and HvT SNP22 (*Ppd-H1*). Four major QTL, and the interactions between them, accounted for most of the variation in both field (71% to 92%) and greenhouse trials (55% to 86%). These were coincident with the location of the major genes for response to vernalization and short photoperiod (*Ppd-H2* on chromosome 1H). A major QTL, near the centromere of chromosome 2H, was the most important under autumn sowing conditions. Although it is detected under all conditions, its action seems not independent from environmental cues. An epistatic interaction involving the two vernalization genes was detected when the plants were grown without vernalization and under long photoperiod. The simultaneous presence of the winter Mogador allele at the two loci produced a marked delay in heading date, beyond a mere additive effect. This interaction, combined with the effect of the gene responsive to short photoperiod, *Ppd-H2*, was found responsible of the phenomenon known as short-day vernalization, present in some of the lines of the population.

Keywords: *Barley, flowering time, photoperiod, QTL, vernalization*

Barley is often grown under semi-arid conditions. Adjustment of crop phenology to the resources available is the main factor for the determination of grain yield in water limited environments (Ludlow and Muchow 1988). Thus, breeding programs for semi-arid conditions must include the attainment of an appropriate heading date among their objectives. Barley growing areas in Spain present mostly a Mediterranean climate characterized by mild to cold winters, with temperatures rising rapidly during spring, hot dry summers, and limited rainfall, usually concentrated in mid-autumn and spring. Producers prefer autumn over winter sowings to benefit from a longer growing period, and to make the most of both rain maxima. The decision on the type of cultivar to grow under these conditions is not straightforward. The choices range from mid- to late-spring cultivars, with some degree of freezing tolerance, to strict winter cultivars with a strong vernalization requirement, depending on the frequency of occurrence of harsh winters (which follows geographic clines). To make informed decisions on the type of cultivars suited to each specific situation, breeders need to have detailed knowledge on the genetic factors affecting barley development in Mediterranean environments.

It has long been known that vernalization requirement (Takahashi and Yasuda 1971), photoperiod response (Roberts et al. 1988) and earliness *per se* genes (Gallagher et al. 1991) are the main factors controlling heading date in barley. Laurie *et al*. (1995) located several major QTL governing these traits on a barley genetic map. Previous QTL studies were made using neutral markers. Currently, there exist allele-specific markers for some candidate genes controlling these processes (Turner et al. 2005; von Zitzewitz et al. 2005; Faure et al., 2007, Szűcs et al. 2007).

Considering the results of previous studies (Casas et al. 1998; Igartua et al. 1999), the cross between cultivars Beka and Mogador was selected to carry out this study. The

parents showed molecular polymorphism at several of the main regions controlling vernalization and photoperiod response.

The aims of this work were: i) to determine the most decisive genetic factors for heading date under Northern Spanish (Mediterranean) conditions, and ii) to assess the effectiveness of closely linked markers and candidate genes, to explain genetic variation in a mapping population grown under Northern Spanish environmental conditions. This is the first step of a larger study on diversity of genes and QTLs controlling heading time in barley under Mediterranean conditions, and their effect on adaptation and yield.

Material and methods

Plant material

A population of doubled-haploid (DH) lines of the spring x winter cross between the French two-row cultivars Beka (Bethge XIII x Kneifel) and Mogador (Alpha x Sonja) was used. This population was derived via anther culture from the F_1 of the cross in the framework of the Spanish Barley Breeding Programme. Overall it showed acceptable yield and good agronomic characteristics for the region.

From an original set of 228 lines, a smaller subset of 120 was selected, according to the results of 6 small-plot field experiments carried out prior to this study. Plants were clustered in 5 groups, according to similarities of their heading dates to the behaviour of cultivars with different phenological response. The subset of 120 lines was chosen from the 5 groups, taking into account their proportion in the original set, but imposing a minimum of 15 plants from each group.

Phenotyping

The study was mainly focused on autumn sowings, the most frequent in the area, but other experiments were also performed under contrasting field conditions (winter and spring sowings), and under greenhouse controlled conditions (four different combinations of temperature and photoperiod), to provide assessment of heading date under contrasting field conditions of photoperiod and temperature.

Three autumn-sown trials were carried out at three locations in Northern Spain: Zaragoza, Valladolid and Huesca (latitudes around 41.5ºN) during three seasons (2001-2003). Experimental design at each location was an alpha lattice with three replicates. These trials were coded as AUZA01, AUVA02 and AUHU03, respectively. Three additional trials were carried out at Valladolid, in winter sowing in 2001 (WIVA01), and spring sowing in 1999 and 2001 (SPVA99 and SPVA01). These trials were unreplicated.

For all field trials, plots consisted of six or eight rows, 6 m long, and between 1.2 and 1.5 m wide, depending on locations. Crop husbandry followed local practices at each location. Days to heading were calculated as the number of days between the 1st of January and the day when approximately 2 cm of awns were visible in 50% of stems. Environmental conditions of each trial are detailed in supplementary Table 1. Day length calculation includes periods of twilight (Slafer and Whitechurch 2001).

The population was also tested under controlled conditions in an experiment that combined presence or absence of vernalization, with long and short photoperiod. Treatments were named V_LP (vernalization followed by long photoperiod), V_SP (vernalization followed by short photoperiod), NV_LP (no vernalization, long photoperiod), and NV_SP (no vernalization, short photoperiod), and were applied as follows: For the V_LP and V_SP treatments, four plants per genotype were vernalized

for 8 weeks in a growth chamber at 4ºC during light time and 9ºC in the dark, with 14 h light (V_LP) or 9 h light (V_SP). When the vernalization period was completed, two plants per genotype were transferred to glasshouses with day length set to 17 h (V_LP) or approximately 10 h (natural day length, labelled as short photoperiod, V_SP), and temperature set to 20/10ºC (day/night). Two weeks before the end of the vernalization period, another four seeds per cultivar were sown in pots, directly within the long and short photoperiod glasshouses (NV_LP and NV_SP treatments, respectively). By the end of the vernalization period, both vernalized and unvernalized plants reached approximately the same developmental stage. One plant was tested for each genotype-treatment combination. Final leaf number (FLN)on the main stem was recorded for each plant. Cooling degree-days (CDD) were calculated as the sum of daily differences between the average temperature and 12ºC, when average temperature was below 12ºC. A maximum of 9ºC was considered for days with average temperature lower than 3ºC, since there is no increase of the vernalization effect below this temperature (Trione and Metzger 1970).

Vernalization requirement (Ver_LP) was estimated for each line as the difference in number of main stem leaves between the NV_LP and the V_LP treatments. Photoperiod sensitivity (Pho_V) was calculated as the difference in the number of leaves between the V_SP and the V_LP treatments.

Genotyping

Genomic DNA was extracted from young leaf tissue of greenhouse-grown plants as described by Casas et al. (1998). Genotyping was carried out with 215 markers: 10 RFLP, 5 STS (2 from candidate genes), 15 RAPD, 112 AFLP and 73 SSR (15 ESTs and 58 genomic-derived markers). The RFLP loci were named using standard North

American Barley Genome Project (NABGP) nomenclature. RFLP probes converted to STS primers were also utilized (Blake et al. 1996; Künzel et al. 2000). Lowercase letters were employed to differentiate them from the RFLP loci. The RAPD loci were mapped using OPERON primer sets. The AFLP markers (EcoRI/MseI) were analyzed following the instructions supplied with the Invitrogen AFLP kit. The AFLP loci were named according to Qi and Lindhout (1997) and Waugh et al. (1997). The SSR loci were identified according to their nomenclature in the literature (Liu et al. 1996; Dávila et al. 1999; Pillen et al. 2000; Ramsay et al. 2000; Moralejo et al. 2004).

Vernalization candidate genes, HvBM5 (*Vrn-H1*) and HvZCCT (*Vrn-H2*) were evaluated as reported by von Zitzewitz et al. (2005). Differences in size at the first intron of HvBM5A were tested: the spring allele (Beka) was analyzed with primers HvBM5.55F and HvBM5.56R; the winter allele (Mogador) was assayed with primers HvBM5.88F (5'-gaatggccgctactgcttag-3') and HvBM5.85R (5'-tctcataggttctagacaaagcatag-3'), or primers HvBM5.66F (5'-ctttagctgttcgacggagg-3') and HvBM5.67R (5'-ctacgccgagcacagaaagc-3'), all within the large size intron.

Linkage map construction was performed using JoinMap 3.0 (van Ooijen and Voorrips 2001). In each chromosome, markers which caused a normalised difference in goodness-of-fit chi-square higher than 5 units after their addition were excluded from the map. Map distances are given in centiMorgan (Kosambi function).

QTL analysis

QTL analysis was performed using the composite interval mapping (CIM) procedure (Zeng 1994) implemented in Windows QTL Cartographer 2.5 (Wang et al. 2005). Up to 21 cofactors for CIM were chosen using a stepwise regression procedure with a significance threshold of 0.05. Walk speed was set to 2 cM, and the scan window

to 10 cM beyond the markers flanking the interval tested. Experiment-wise significance (α=0.05) likelihood ratio test (LR) thresholds for QTL identification were determined with 1000 permutations, and expressed as LOD (LOD = 0.217 LR). Epistatic interactions between QTL were evaluated with the Multiple Interval Mapping (MIM, Kao et al. 1999) tool implemented in Windows QTL Cartographer using Bayesian Information Criteria (BIC-M0).

Heading date or FLN values used for QTL analysis were calculated using adjusted line means (weighted least square means) for each experiment. The proportion of the total variance explained by the QTL was calculated as the coefficient of determination of the multilocus model for each experiment using MIM. Analyses of variance and regression analyses with markers linked to the QTL were performed using the GLM procedure of SAS v9 (SAS Institute Inc., Cary, NC, USA).

Tests for QTL x Environment interactions were performed using NQTL (Windows version of MQTL, Tinker and Mather 1995) at an experiment-wide significance level of 0.05 and 43 background markers.

Results

Linkage map

A linkage map with 215 markers was constructed. Map density for QTL analysis was reduced to a minimum of 1.5 cM between markers, by removing co-segreganting markers (similarity higher than 0.95) and those with poor goodness of fit. The final map for QTL analysis had 126 markers distributed over 7 linkage groups (Fig. 1) at a LOD score of 5.0. All linkage groups were assigned to barley chromosomes. The linkage map

covered 1,163 cM, with an average distance of 9.2 cM per marker. Markers were distributed across the entire genome, except on the distal part of the short arm of chromosome 1H, where no polymorphic markers were found. Segregation distortion was significant ($P<0.05$) in favour of Beka alleles in regions of chromosomes 1H (E35M48_i - Bmag382), 3H (E36M48_j - OPAN02), 4H (E35M47_l) and 6H (E37M61_d - Bmag173), and in favour of Mogador alleles in chromosomes 2H (E35M47_h - E37M48_f), 5H (scssr07106 - Bmac096 and HVDHN7 - HvBM5) and 7H (E37M47_d - E41M47_a).

Heading time

Frequency distributions of days to heading for field trials and number of leaves for greenhouse treatments showed a quantitative response and transgressive segregation (Fig. 2).

Environmental conditions of the trials carried out in this study were very diverse (supplementary Table 1). Lack of enough CDD quite possibly led to insufficient vernalization (trials SPVA01 and SPVA99). These treatments, along with greenhouse treatments NV_SP and NV_LP resulted in large heading date differences between the parents, and a wide and flat distribution of DH heading dates (Fig. 2).

All correlation coefficients between field and greenhouse experiments were significant (Table 1). The highest correlation coefficients were obtained between spring–sown field trials and the greenhouse treatment NV_LP. A high correlation coefficient was also obtained between autumn-sown trials and the V_SP treatment. These correlations are consistent with expectations, based on CDD and hours of daylight at heading time measured at the field trials (supplementary Table 1).

Quantitative trait loci, main effects

Several QTL for heading date were found in the field and greenhouse experiments (Fig. 3). The amount of phenotypic variation explained jointly by the QTL ranged from 81% to 93% for the field trials, and from 59% to 86% for the greenhouse trials (Fig. 3). Four major QTL (Fig. 3), and the interactions between them, accounted for most of the variation in both field (71% to 92%) and greenhouse trials (55% to 86%).

An analysis of variance, including the nearest markers to the QTL peaks as sources of variation, and their interactions, is shown in supplementary Table 3. QTL positions and confidence intervals are shown in supplementary Table 2.

In autumn-sown experiments, a QTL near the centromere of chromosome 2H (bin 8) was the most significant. It was located in the interval E36M61_a – OPAS05, with the peak at Bmac132, in the vicinity of gene *Eam6*. In all cases, the Beka allele conferred later heading. Another large effect QTL was found in the long arm of chromosome 1H (bin 12 -13), in the interval MWG518 – E35M47_b, with the peak at Bmag382, in the vicinity of gene *Ppd-H2*. The Mogador allele conferred late heading. Under greenhouse conditions, it had strong effects under short photoperiod, and a small effect at the V_LP treatment. The third most relevant heading date QTL at all field trials was on chromosome 7H (interval MWG089 – EBmac521b), with Mogador as the later allele.

Other QTLs, although with minor effect compared to the previous ones, were also detected in autumn sowings (Fig. 3), on chromosomes 2H (at two field trials), 3H (one at three field trials, another one only at AUVA02), 4H, 5H, and 6H (all in just one field trial).

Regarding late sowings, the most important QTL coincided with the positions of markers HvZCCT and HvBM5 in chromosomes 4H and 5H, respectively

(supplementary Table 3). These positions co-locate with the vernalization genes *Vrn-H2* and *Vrn-H1*, respectively, whose allelic variations determine the requirements of vernalization in temperate grasses (Yan et al. 2003, 2004; Fu et al., 2005; von Zitzewitz et al. 2005; Szűcs et al. 2007). In autumn-sown field trials, no effect of these genes was detected (Fig. 3a). As the sowing date advanced, the effect of these markers grew progressively, being smaller at the winter sowing (WIVA01), and larger at the spring-sown trials. Other lesser effect QTL were found in chromosome 3H (peak at 116 cM), and only in the winter-sown trial in chromosomes 3H (peak at 170 cM) and 7H (peak at 12 cM).

Under controlled conditions, the effects of the QTL at Bmag382, Bmac132, HvZCCT and HvBM5 were very evident. Three other minor QTL were detected in chromosome 5H, at different treatments, and one in chromosome 6H, on the same location as for the AUVA02 field trial. Regarding the Ver_LP effect, the only loci affecting this trait were HvZCCT and HvBM5 and their interaction (Fig. 3b, supplementary table Table 3). Allelic differences at markers Bmag382, Bmac132, HvZCCT, and (to a lesser extent) Bmag812 were associated to the Pho_V effect.

Interactions between QTL

Some significant interactions between QTLs were found (Fig. 4 and supplementary Table 3). The main interaction was found between *Vrn-H1* and *Vrn-H2* in spring-sown field trials, in the NV_LP treatment, and in the VER effect (interaction HvZCCT*HvBM5). The simultaneous presence of the winter Mogador allele at the two loci produced a marked delay in heading date, beyond a mere additive effect. There was also a significant QTL x Environment effect for these two regions (Fig. 3a).

This interaction is further described in Fig. 4, where results have been split into the four classes defined by the combinations of HvZCCT and HvBM5. The allelic composition at the two main vernalization genes was used by von Zitzewitz et al. (2005) to define growth type classes: 'spring' when the plants lack the winter allele in *Vrn-H1* (HvBM5), 'facultative' if *vrn-H1* winter allele is present but not its repressor *vrn-H2* (HvZCCT), and 'winter' with both *vrn-H1* and *Vrn-H2* winter alleles functional. We will follow these denominations from now on, bearing in mind that 'spring' actually comprises two classes *(Vrn-H1/Vrn-H2* and *Vrn-H1/vrn-H2*). No differences among classes were found at the autumn-sown trials. Differences were small, though significant, at the winter-sown trial, and rather large at spring-sown ones. In these, 'spring' classes headed at the same time, and significantly earlier than the 'winter' class, 'facultative' being in an intermediate position. In the greenhouse treatments, there were no differences under the less inductive conditions (NV_SP). The two vernalization treatments produced a pattern rather similar to the winter-sown field trial, and at the NV_LP the four classes behaved similar to the spring-sown trials.

To detect possible repressors, other than *Vrn-H2*, making the facultative class later than the spring one in the spring-sown trials, we performed analyses of variance for each spring-sown trial, within each of the vernalization classes (*spring, facultative*, and *winter*). We used the multilocus model that included the nearest markers to the significant peaks detected in the CIM at each experiment, and their significant interactions. Then, the rest of the 126 markers were sequentially added and removed to this model, one by one, (data not shown). In this manner, five markers with a consistent significant effect on heading date only for the facultative class lines in the two spring-sown experiments were detected. Three of them were just above the significance threshold using a False Discovery Rate (Benjamini and Hochberg, 1995) approach at

the chromosome level. The other two presented a much larger effect, and peaked at markers just beside heading time QTL found for the autumn-sown trials (and within the 2-LOD confidence interval). These were OPAN02 in bin 8 of chromosome 3H and MWG089 in bin 3 of chromosome 7H (Table 2). In all cases the Beka alleles caused early heading (Table 2) and no interaction was found between them.

Also under spring sowing conditions, we found an interaction between Bmac132 (from now on, *Eam6*) and *Vrn-H1* (supplementary Table 3). In both experiments, lines carrying the Beka allele in *Eam6* headed significantly later when the winter allele of *Vrn-H1* was present. Under controlled conditions, *Eam6* also presented significant interactions with vernalization genes, but more so with *Vrn-H2* (HvZCCT), evident at the V_SP treatment. Other significant interactions found were of lesser importance.

We also observed some results in the controlled conditions experiment which agreed with a phenomenon previously described as *short-day vernalization* by Roberts et al. (1988), in which exposure of winter genotypes to short-photoperiod conditions could substitute the effect of vernalization. This is apparent in Fig. 5, as the plants carrying Mogador alleles at the two vernalization loci, and the Beka allele at Bmag382 (from now on, *Ppd-H2,* for simplicity), produced a significantly lower number of leaves until heading at the NV_SP treatment than at the NV_LP one (both marked with an asterisk in Fig. 5).

Actually, the average number of leaves for lines with Beka alleles at *Ppd-H2* was rather similar across all classes of lines (*spring, facultative* and *winter*), at the NV_SP treatment, and the same could be said for the Mogador allele, though it was 2-3 leaves more than Beka overall (Fig. 5).

There was a significantly higher number of leaves for the *winter* class (Mogador at both vernalization loci) at the NV_LP treatment. This increase was evident for both

Ppd-H2 alleles (NV_LP bars at the M/M class in Fig. 5). But the effect it produced for the Beka allele at *Ppd-H2*, when compared with the number of leaves shown by the same lines at the NV_SP treatment, was an apparent shortening of the cycle of unvernalized winter lines, due to the short photoperiod. For other marker combinations, NV_SP was always the treatment where more leaves were produced.

A small quantitative interaction was found between *Ppd-H2* and *Vrn-H1* in non-vernalized plants (supplementary Table 3) and we also found that the effect of *Ppd-H2* was larger in the non-vernalized treatment (Fig. 3b). But, no significant three-way interaction among markers for the three loci (*Vrn-H1, Vrn-H2* and *Ppd-H2)* was found.

Discussion

Distances and marker positions estimated for Beka x Mogador were consistent with other published barley genetic maps (Ramsay et al. 2000; Marquez-Cedillo et al. 2001; Francia et al. 2004).

Major QTL

Genetic control of flowering time in barley has been thoroughly studied and it has long been established that vernalization requirement (Takahashi and Yasuda 1971) and photoperiod sensitivity (Laurie et al. 1994, 1995) are the most important factors controlling this trait.

Four major QTL, whose positions agree with previously reported genes, were found in this study, at the following locations:

i) Marker Bmac132, on the centromeric region of chromosome 2H (bin 8, bin classification after Kleinhofs and Han 2002), coincident with the earliness *per se* locus *eps2S* (Laurie et al. 1995), whose effect is evident under spring and autumn sowing conditions. It is also coincident with the early maturity locus *Eam6*, which confers early heading under both long- and short-day conditions (Franckowiak and Konishi 2002; Horsley et al. 2006). This was the most important locus for heading time under autumn sowing conditions in our study, and had also a large effect in spring sowings.

The centromeric region of chromosome 2H has been consistently identified in studies searching for heading date QTL, but with large differences in allelic effects. It was first described by Laurie et al. (1994), who reported a large effect of this QTL on heading date (around four days), though the method of analysis available at that time prevented a good estimation of its effect independent of the linked locus *Ppd-H1*.

For *spring x spring* populations, evaluated in spring sowings, the difference between alleles found in a rather large series of studies was always between 1 and 3 days (Qi et al 1998; Marquez-Cedillo et al. 2001; See et al. 2002; Mesfin et al. 2003; Canci et al. 2004; Dahleen et al. 2004; Horsley et al. 2006), whereas for studies involving autumn sowings, its effect was always above 4 days, either in *spring x spring* (Tohno-oka et al. 2000; Boyd et al. 2003; Moralejo et al. 2004) or in *spring x winter* crosses (Laurie et al. 1994; Read et al. 2003). In this last case (population Sloop/Halcyon), the difference between alleles was 6 days, similar to the one found in our study, also a *spring x winter* cross. *Eam6* was identified as the main locus responsible of heading date under Spanish conditions in the population Beka x Logan (Moralejo et al. 2004), and had a large effect in Australian conditions (also a Mediterranean climate), under both short and long photoperiods (Boyd et al. 2003).

The only exceptions to this trend found in the literature were two populations involving a cross with the same *Hordeum spontaneum* accession, evaluated in spring sowings. In these two cases (Pillen et al. 2003, 2004), differences between alleles at this QTL were 9.6 and 12.6 days, with the early allele contributed by the *H. spontaneum* parent, which could be different from the alleles found in barley cultivars.

Eam6 (or *eps2s*) has been defined as an *early maturity* gene, but there is no consensus about its dependence on environmental conditions: it has been described either as photoperiod-insensitive (Boyd et al. 2003) or as photoperiod sensitive (Franckowiak and Konishi 2002; Horsley et al. 2006). Our data support the effect of this locus under a wide variety of conditions, but with the size of its effect modulated by environmental cues that could be photoperiod (an effect on photoperiod sensitivity was reported in Fig. 3) or temperature (we also observed interactions of this locus with the markers for the two vernalization genes, supplementary Table 3). It is interesting to note that the effects reported for this locus in the literature, for experiments carried out at lower latitudes and/or early sowings, have always been large; whereas experiments at higher latitudes and/or late sowings always detected a QTL of lesser effect. Consistently with this hypothesis, an AB-QTL study by Talamé et al. (2004) found increasing effects of heading date in a spring x *H. spontaneum* cross (Barke/HOR11508), with large effects (8-9 days) in autumn sowings in north Africa, and lower effect (3.2 days) in a late sowing (February, Italy).

Ppd-H1, also in the short arm of chromosome 2H, has been reported as the main gene affecting heading time in barley under photoperiods of 13 hours or longer (Laurie et al. 1994, Turner et al. 2005). We tested the functional polymorphism in SNP22 of *Ppd-H1* (Turner et al. 2005), and found no polymorphism; therefore, it is likely that both parents of the population carried the same, non-responsive allele. Thus, lack of

polymorphism for *Ppd-H1* in this population prevents its study but, on the other hand, allows a clearer resolution of the effect and interactions of *Eam6*, which could have been partially masked by its linkage to *Ppd-H1*, as may have happened in populations segregating for both major QTL on chromosome 2H, such as Steptoe/Morex (Hayes et al. 1993) and Igri/Triumph (Laurie et al. 1994, 1995).

ii) Long arm of chromosome 1H (bin 12-13), marker Bmag382, coinciding with the position of the photoperiod response gene *Ppd-H2*, which causes differences in heading date under short day conditions in field and greenhouse (Laurie et al. 1995; Francia et al. 2004). Its effect was also most influential under Australian field conditions, where cultivars are also exposed to short photoperiods during most of the growing season (Boyd et al. 2003). In our experiments, lines carrying the Mogador allele were always delayed at field autumn sowings, V_SP, and NV_SP treatments, in which most of the vegetative phase elapses with photoperiods around 10 – 11 hours.

iii) Two other main QTLs were found under long photoperiod and non-vernalizing conditions, in the field and the greenhouse, coinciding with the positions of *Vrn-H1* (marker HvBM5, bin 11 in chromosome 5H) and *Vrn-H2* (marker HvZCCT, bin 13 in chromosome 4H). Allelic variations and interactions of both loci determine the requirements of vernalization in temperate grasses (Yan et al. 2003, 2004; Fu et al. 2005, von Zitzewitz et al. 2005, Szűcs et al. 2007).

Significant segregation distortion was found in the *Ppd-H2* and *Vrn-H1* regions. We tested the markers at the distorted regions for a remnant of 108 DH lines of this cross, not used in this study, and found the same distortion which was, therefore, not due to sampling.

Minor QTL

The positions for most of the minor QTL were also coincident with previously reported ones. Only one minor QTL was found in both field and greenhouse conditions, coincident with the position of *eps6L* in bin 7 of chromosome 6H (Laurie et al. 1995). Though it had a more general effect, the largest effect was apparent in the greenhouse under short photoperiods, as in the current study (supplementary Table 3). There is also a report of e*ps6L* effect, but with vernalized plants under long photoperiods (Boyd et al. 2003).

Other minor QTLs were found only in the field experiments. The confidence interval of the one detected around 138 cM on chromosome 2H, overlaps with two QTL found in spring barley (Hayes et al. 1993; Pillen et al. 2003). The confidence interval of the QTL found in chromosome 7H spans over bins 3-5 and overlaps with the QTL for heading date *eps7s* (Laurie et al. 1995), which had effect only in spring sowings. Marquez-Cedillo et al. (2001) found a similar effect in the same region (bins 3-4). Pan et al. (1994) also reported a QTL in this region (bins 4-5), in winter sowing conditions. As shown below, loci in this region were associated with differences in heading time also in spring conditions, but only in facultative plants.

Three of the QTLs found in chromosome 3H also agree with other previously reported loci: Hayes et al. (1993) found a QTL in bin 8 in autumn sowings, as our QTL at Bmag225; Mesfin at al. (2003) found another one in bin 12-13 in spring sowing, similar to our QTL with peak at E35M47_k (supplementary Table 3); and Gallagher et al. (1991) found a third one in bin 15-16 coincident with the position of our QTL with peak at HvM62, and locus *eam10* (Börner et al. 2001; synonymous of *easp* in Gallagher and Franckowiak 1997, and *eps3L* in Laurie et al. 1995), with effect in short day photoperiods. It is also coincident with other QTLs for heading date reported in field

autumn sowing and 8 hour-photoperiod greenhouse vernalized and non vernalized plants (Pan et al. 1994).

On chromosome 5H, the QTL in bin 6 (peak at scssr15334) agrees with the position of *eps5L* (Laurie et al. 1995), found both under greenhouse and field conditions, under photoperiods longer than 13 hours. The QTL found in bins 8-9 (peak at Bmag812) was in the same position as one found in an autumn-sown field experiment by Pan et al. (1994). The QTL in bin 10 has not been previously reported, but its detection in unvernalized plants grown under short photoperiod, and its proximity, suggests that it could be *Vrn-H1*.

The paramount role of vernalization genes in the Beka x Mogador population

It seems that the autumn sowing conditions provided periods of low temperature long enough to satisfy vernalization requirements in winter genotypes. This caused a narrower range of variation for heading time in the population, compared to the range of variation observed under spring-sown conditions (Fig. 2), in which vernalization requirements for many lines were apparently not met. Therefore the effect of *Vrn-H1* or *Vrn-H2* was not detected under normal (autumn) sowing conditions, and was mostly evident in spring-sown trials, or in unvernalized treatments in the greenhouse.

The functioning of the two vernalization genes is a clear example of epistasis (Laurie et al. 1995; Yan et al. 2004; Karsai et al. 2005; Koti et al. 2006; Trevaskis et al. 2006; Szücs et al., 2007). In the spring-sown experiments and at the NV_LP treatment (under long photoperiod and almost absence of vernalization) *Vrn-H1* is repressed by *Vrn-H2* and, therefore, 'winter' lines were remarkably delayed with respect to spring and facultative lines (Fig. 4). This delay of winter genotypes under non-inductive conditions was confirmed when analyzing the number of leaves in the NV_LP

greenhouse treatment (Fig. 4). Our results also confirmed another aspect of the hypothesis put forward by Trevaskis et al. (2006), i.e., that r*epression of Vrn-H1 by Vrn-H2 is ineffective when vernalization requirements are fulfilled, and also under short day conditions irrespective of the vernalization.* This was apparent at the autumn-sown trials, and at the short photoperiod treatments (V_SP, NV_SP) in which all vernalization classes reached heading almost at the same time (Fig. 4). Only when there was concurrent lack of vernalization and long photoperiod, the effect of repression of *Vrn-H2* on *Vrn-H1* was detectable.

However, we have found that the effect of *Vrn-H2* is not restricted to long day conditions only. There was a QTL at the *Vrn-H2* region in the V_SP treatment (Fig. 3b). We saw that plants with the winter allele in *Vrn-H2* headed earlier, irrespective of the *Vrn-H1* allele, when exposed to vernalization and short photoperiods (treatment V_SP in Fig. 4).

Interactions between photoperiod and vernalization genes, although significant (supplementary Table 3), do not seem very important for the determination of heading time under our field conditions. One of these interactions could be responsible for the phenomenon referred to in the literature as *short-day vernalization,* as mentioned in the *Results* section. Laurie et al. (1995), and Igartua et al. (1999) suggested a role of *Ppd-H2* in combination with the two main vernalization genes to explain this phenomenon. Laurie et al. (1995) observed that genotypes with a strong vernalization response under long photoperiod conditions, and carrying a particular allele on *Ppd-H2*, headed at the same time as genotypes that lacked vernalization response when both types were grown under short photoperiods (10 hours). This fact suggests that short day could substitute for cold treatment to hasten heading of these winter genotypes. A similar situation was described by Roberts et al. (1988) for the cultivar Arabi Abiad, which showed an

apparent shortening of time to heading in unvernalized plants, when grown under short photoperiod, which was equivalent to about half of the effect due to vernalization. Actually, what we found is that the additive effect of the *Ppd-H2* (Beka allele always earlier than Mogador, under short photoperiod, Fig. 5) with the *Vrn-H1*Vrn-H2* interaction (delay of the M/M class at the NV_LP treatment, Fig. 5) produces an apparent *short-day vernalization* effect, which resembles closely the findings of previous authors, i.e., the fact that *winter* (M/M) lines with the Beka allele at *Ppd-H2,* grown without vernalization, have lower number of leaves under short photoperiod conditions (NV_SP) than under long photoperiod (NV_LP), whereas spring and facultative have more leaves (Fig. 5).

The Beka and Mogador alleles of *Ppd-H2* are possibly the same allele presented by cultivar Triumph and Igri, respectively, in the study by Laurie et al. (1995), according to the RFLP marker MWG518, and the derived STS marker aMWG518, which present the same polymorphism for each pair of cultivars (Beka/Triumph, Mogador/Igri). The behaviour of cultivar Arabi Abiad in Roberts et al. (1988) study was actually very similar to the behaviour of the most extreme lines in population Beka x Mogador, attending to the number of leaves reported for this cultivar at the treatments most similar to ours.

We searched for other possible interactions with the two main vernalization loci. In spring sowing conditions, *facultative* lines should reach heading at the same time as the spring lines, since they have the winter allele of *Vrn-H1* but lack the repressor *Vrn-H2,* according to the model by Trevaskis et al. (2006). However, facultative lines headed around 10 days later than spring lines in each spring-sown experiment (Fig. 4). Therefore, we propose the hypothesis that there could be other loci interacting with *Vrn-H1*, other than *Vrn*-H2, that could be repressing its action.

Other repressors of *Vrn-H1* have been proposed as *HvVRT2* (Kane et al. 2005), detected on chromosome 7H, in the *facultative* photoperiod-responsive cultivar Dicktoo when exposed to a photoperiod of 8 hours. We did not have comparable conditions in our experiments. Nevertheless, we tested the *HvVRT2* genotype with the same CAPS marker as Szűcs et al. (2006). Both parents showed the same allele, similar to Dicktoo, and thus we could not expect to find a QTL in this region. But we can expect a repressive action of this *HvVRT2* allele (if it is similar to the one from Dicktoo) on *Vrn-H1* in all the population, which could explain, at least partially the intermediate behaviour of *facultative* lines described above.

The results suggest that there could be minor repressors of *Vrn-H1* in two regions: in bin 8 of chromosome 3H, close to marker OPAN02, and in bin 3 of chromosome 7H, close to marker MWG089 (Table 2), which become evident only when *Vrn-H2* is not present. In both cases the Beka allele caused early heading but their effect is still short of accounting for all the delay of the *facultative* lines described above. We cannot tell whether the addition of the effects of these two loci with the effect of the *HvVRT2* repressor is enough to account for the delay.

Beka x Mogador has been confirmed as an excellent population for vernalization study purposes since it shows polymorphism for both *Vrn-H1* and *Vrn-H2*, and has been genetically characterized with allele specific markers for these genes, which provided an excellent resolution for the detection of the effects of these genes and their interactions, which included a plausible explanation for the phenomenon of *short-day vernalization*.

We have been able to identify the QTLs that explain most of the variation in flowering time of barley in Northern Spanish autumn sowing conditions and we have contrasted and validated these results with field experiments in other sowing times and

under controlled conditions of vernalization and photoperiod in the greenhouse. The amount of variation explained by only four allele-specific or closely linked markers to these genes, some of them proposed by the first time in this study, was in all cases over 75%, and we could identify the independent sources of variation making flowering time highly predictable and almost a qualitative trait. The QTL at the centromere of chromosome 2H deserves further study, as one of the crucial loci controlling heading time under Mediterranean conditions.

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Table 1. Pearson correlation coefficients between days to heading in field experiments and number of leaves in greenhouse treatments.

	SPVA01	SPVA99	WIVA01	AUZA01	AUVA02	AUHU03
NV_SP	0.37***	0.40***	0.56***	0.67***	0.54***	0.68***
NV_LP	0.89***	0.85***	0.42***	0.20*	0.21*	0.22*
V_SP	0.20*	0.28**	0.62***	0.70***	0.69***	0.71***
V_LP	0.51***	0.55***	0.65***	0.57***	0.57***	0.57***

Correlation coefficient significant at *P < 0.05, **P < 0.01, ***P < 0.001

Table 2. Joint interaction of HvBm5 (*Vrn-H1*) and HvZCCT (*Vrn-H2*) with other loci. Average days to heading from 1st of January to heading date of two spring sowing experiments. Lines are classified according to their genotype for the vernalization genes *Vrn-H1* and *Vrn-H2*.

			AN02 (3H)					MWG089 (7H)				
Class	*Vrn-H2*	*Vrn-H1*	No	**B**	No	**M**	Pr>t	No	**B**	No	**M**	Pr>t
Spring	B	B	19	145.5	7	145.6	0.9835	11	144.8	15	146.1	0.2923
Facultative	B	M	17	153.0	15	156.7	0.0017	12	152.6	20	156.0	0.0066
Spring	M	B	17	145.7	6	147.0	0.3874	13	145.6	10	146.5	0.5219
Winter	M	M	19	171.5	20	172.7	0.2683	14	170.9	25	172.8	0.0827

B: Beka; M: Mogador; No.: number of lines

* Probability that T is greater than a critical value of t under the null hypothesis that both means (B and M on the same line) are equal.

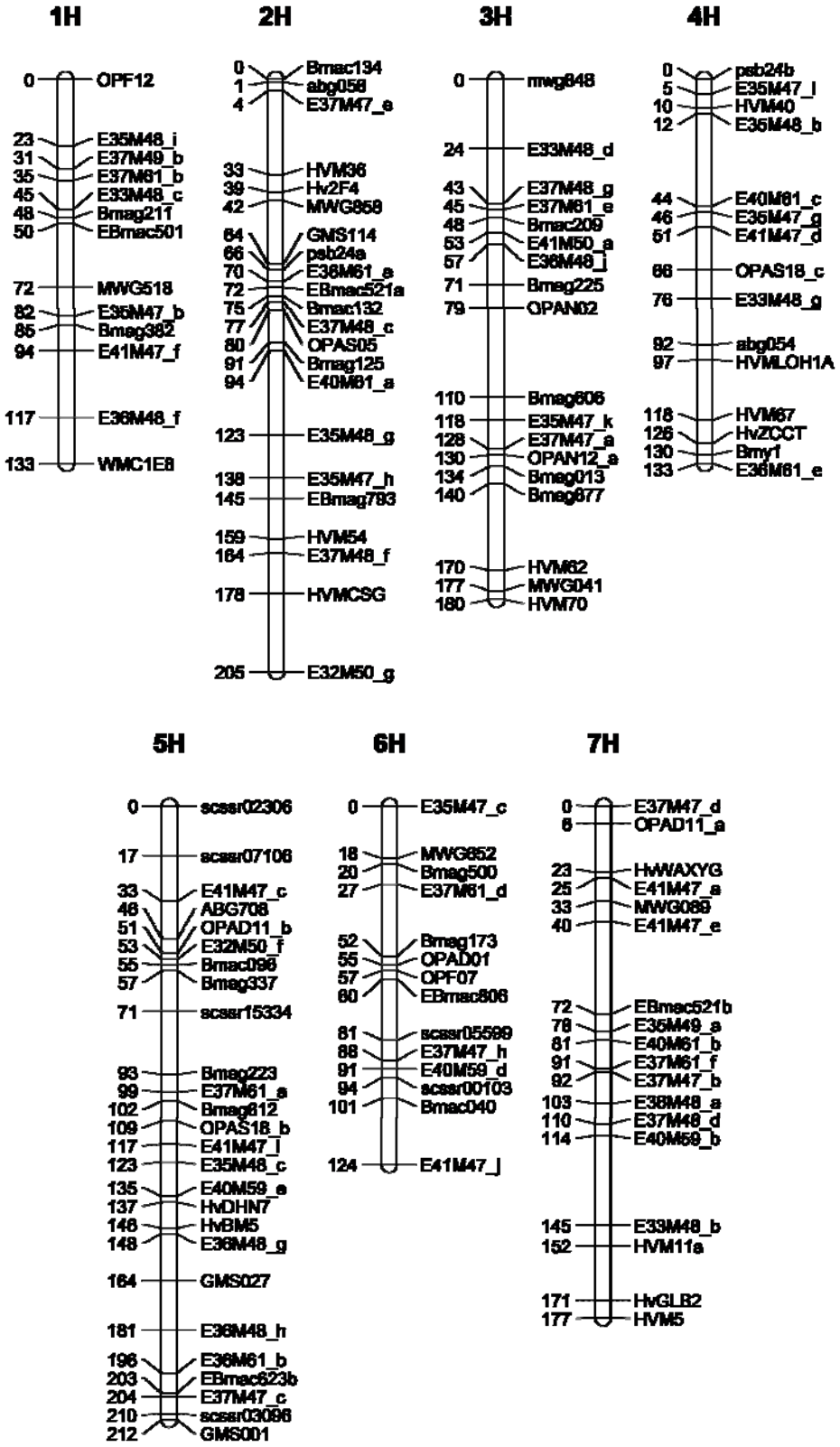


Figure 1. Linkage map of Beka x Mogador doubled haploid population (n = 120). Only 126 spaced markers used for QTL analysis, out of the initial number of 215, are represented (see text). Distances are in Kosambi cM.

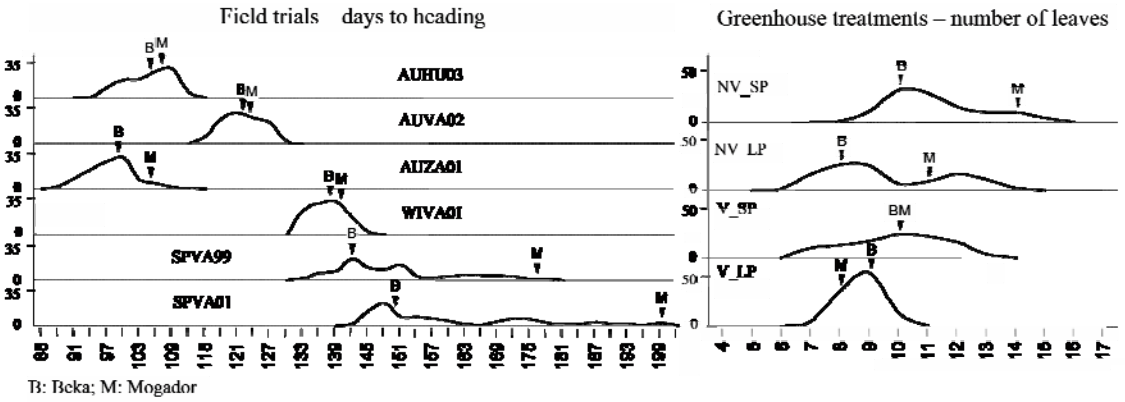
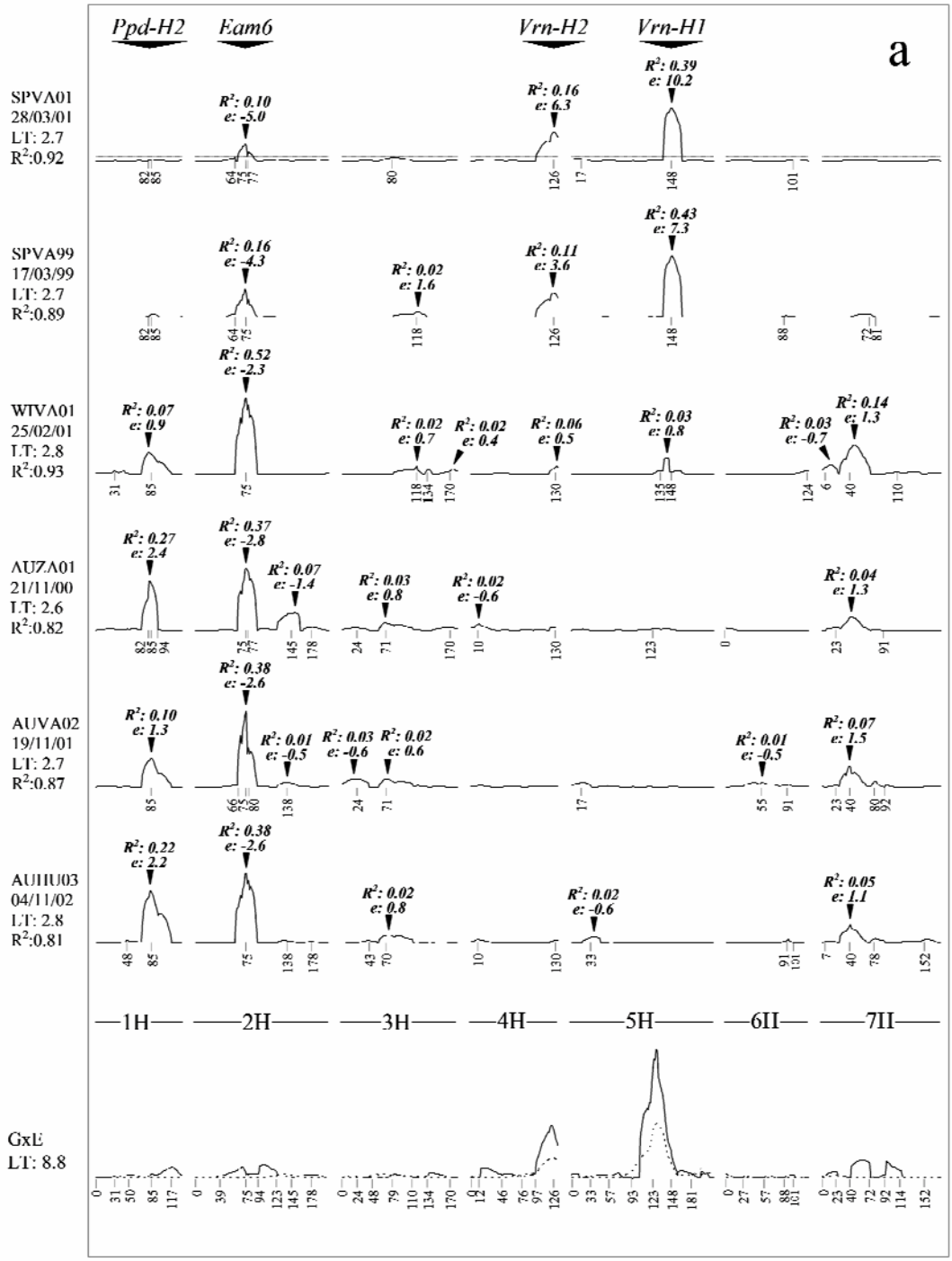


Figure 2. Distribution of genotypes regarding days to heading (field) and number of leaves (greenhouse) of the Beka x Mogador doubled haploid population experiments. The scale in the vertical axis represents relative frequencies of doubled haploid lines, 0 to 35 % in the field trials and 0 to 50% in the greenhouse trials.


Ppd-H2
Eam6
Vrn-H2
Vrn-H1
a
SPVA01
28/03/01
LT: 2.7
R²:0.92
SPVA99
17/03/99
LT: 2.7
R²:0.89
WTVA01
25/02/01
LT: 2.8
R²:0.93
AUZA01
21/11/00
LT: 2.6
R²:0.82
AUVA02
19/11/01
LT: 2.7
R²:0.87
AUHU03
04/11/02
LT: 2.8
R²:0.81
GxE
LT: 8.8
1H
2H
3H
4H
5H
6H
7H

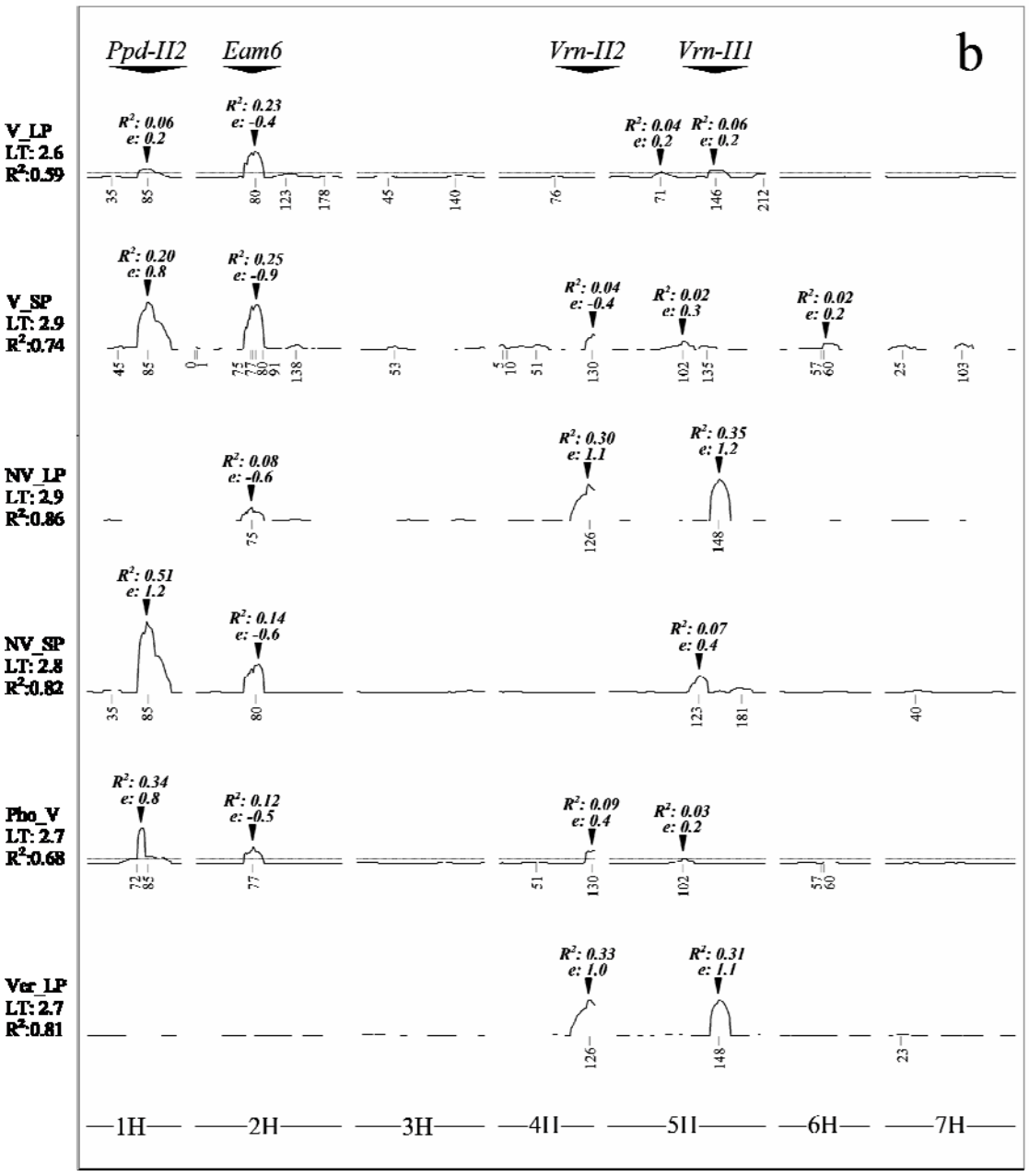


Figure 3. LOD scans of Composite Interval Mapping (CIM) for a) field trials (days to heading) and b) greenhouse treatments and effects (number of leaves). Variable codes are described in the Material and Methods section. On the Y axis, field experiments (3a) are arranged according to sowing date. Also indicated are the code of the experiment, the LOD thresholds (LT) for individual QTL detection based on a experiment-wise error of α=0.05, calculated with 1000 permutations (dotted line), and the R_2 of the multilocus model that includes all significant markers and interactions. For every QTL detected, its individual R^2 and additive effect are indicated. Positive effects indicate later heading of plants carrying

the Mogador allele. Chromosomes 1H through 7H are displayed on the X axis, from left to right, showing the positions of the markers included as cofactors in the CIM analysis. The values of the Genotype x Environment Interaction LOD scans cannot be compared with the ones in the individual trait analysis, since the calculation method is different. Scans for Simple Interval Mapping (SIM – dotted line) and Simplified Composite Interval Mapping (sCIM – full line) are represented (Tinker and Mather, 1995). The LOD threshold in this case corresponds only to the sCIM based on 1000 permutations.

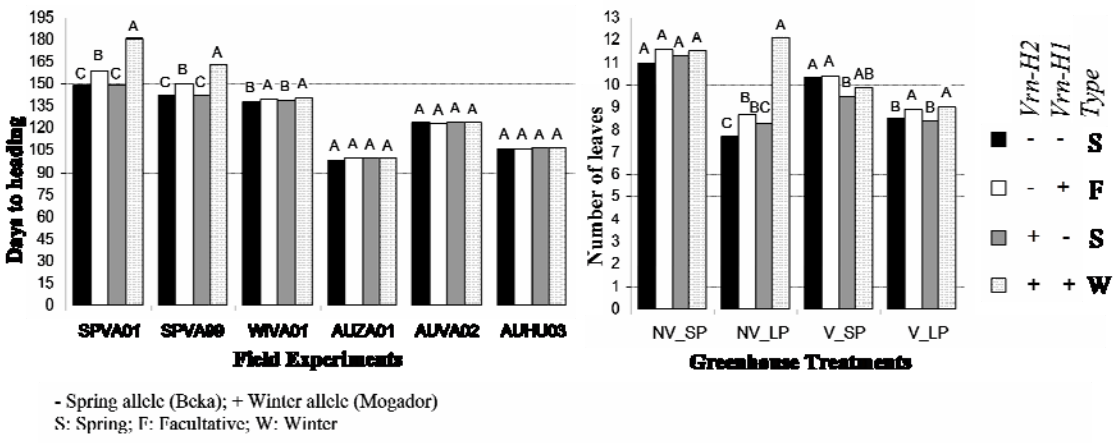


Figure 4. Means of days to heading from January 1st, and number of leaves of the 4 classes of genotypes determined by the allelic composition at *Vrn-H1* and *Vrn-H2*. Due to the strong effect of photoperiod response QTL, means are adjusted for other QTL detected in each experiment. Letters indicate means separation within each experiment. We used the Bonferroni multiple comparison adjustment for the *P*-values and confidence limits for the differences of LS-means with alpha=0.05.

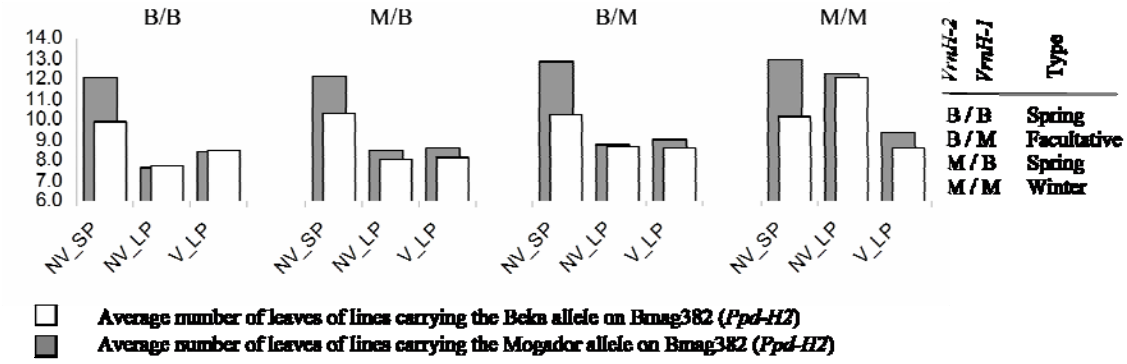


Figure 5. Average number of leaves of the Beka x Mogador DH lines, divided according to their haplotype for the vernalization genes *Vrn-H1* and *Vrn-H2,* and the short photoperiod response gene *Ppd-H2.* The data in this figure explains the effect of *Vrn-H1/Vrn-H2* interaction on the occurrence of short-day vernalization (see text).B: Beka allele; M: Mogador allele.